Data Supplement

Materials and Methods

Animals

Adult male SHR or WKY rats were used for this study. They were housed in temperature- (23 ± 2°C) and light-controlled (lights on between 7 AM and 7 PM) animal quarters and were provided with chow *ad libitum*. Vehicle (water) or valproic acid (VPA, 0.71% wt/vol[1], Sigma) dissolved in water were prepared and provided daily. Another subset of animals (WKY and SHR; n=5 each) were administered hydralazine (HYD, 25mg/kg/day in drinking water[2], Sigma), a direct smooth muscle relaxant and vasodilator, to determine the pressure-independent effect of VPA on cardiac hypertrophy. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental protocol

SHR (n=20) and WKY (n=20) rats underwent echocardiographic assessment at the start and end of the treatment period. Rats received drug or vehicle treatment for 20 weeks. Rats were euthanized at 27 weeks of age. Wet heart and lung weights were measured and analyzed against body weight. Blood and left ventricular tissue (LV) was collected for molecular analysis.

Blood pressure measurement

A tail-cuff plethysmograph (CODA 6 Blood Pressure System, Kent Scientific System, Torrington, CT) was used for blood pressure measurement at baseline, and weekly thereafter. Blood pressure was measured for four consecutive days for determination of weekly average measures. The daily measurements were blindly analyzed for the most closely associated five consecutive runs for use as that day's average value.

Echocardiographic assessment of LV hypertrophy

Echocardiography was obtained at baseline, and repeated at the end of the treatment period. Echocardiogram was performed as described previously[3]. Briefly, transthoracic echocardiography was performed under isoflurane anesthesia, using a Toshiba Aplio SSH770 (Toshiba Medical, Tustin, California) fitted with a PST 65A sector scanner (8 MHz probe) which generates two-dimensional images at a frame rate ranging from 300-500 frames per second. Left ventricular posterior wall thickness at end-systole and end-diastole (LVPWTs and LVPWTd, respectively) was measured digitally on the M-mode recordings and averaged from at least three cardiac cycles.

Assessment of LV hypertrophy through fibrosis staining

Paraffin sections (10μm) were obtained from heart specimens as previously described[4] and stained with picrosirius red for the detection of collagen. The percent area of fibrosis was calculated using ImageJ software (NIH).

Detection of Total ROS and Superoxide ($O_2^{\bullet-}$) in LV heart tissue

One of the most sensitive and definitive methods of superoxide production is electron spin resonance (ESR). In this study, we utilized an established technique for total ROS detection in tissue using ESR and spin traps[3]. Different spin probes were used for the ESR studies. 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) was used to measure the $O_2^{\bullet-}$ levels. All ESR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany).

Sample preparation for ESR studies: The dissected LV tissue from each animal was placed into a 24-well plate containing Kreb's HEPES buffer (KHB) (20mM, pH 7.4). Tissue pieces were then washed twice with the same buffer to remove any trace contamination. Samples were then incubated at 37^{o}C with specific spin probes for 30 minutes.

Total tissue ROS production: Total ROS was determined as previously described[3]. Tissue pieces were incubated at 37^{o}C with CMH (200 μM) for 30 minutes. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS production, under the following ESR settings: field sweep 50 G; microwave frequency 9.78 GHz; microwave power 20 mW; modulation amplitude 2 G; conversion time 327 ms; time constant 655 ms; receiver gain 1 x 10^5. For superoxide production, samples were pre-incubated at 37^{o}C with PEG-SOD (50 U/ml) for 30 minutes, then CMH (200uM) for an additional 30 minutes. Aliquots of the incubated probe media were taken in 50 μl glass capillary tubes for determination of total superoxide production. Addition of PEG-SOD to CMH allowed competitive inhibition of the $O_2^{\bullet-}$-CMH oxidation reaction by the quenching of $O_2^{\bullet-}$ radicals. Since it is cell permeable, PEG-SOD can competitively inhibit the CMH-$O_2^{\bullet-}$ interaction both intracellularly and extracellularly, thus allowing accurate measurement of total tissue $O_2^{\bullet-}$ production. To determine actual total tissue superoxide production, the values obtained from incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only.

RNA isolation and real-time RT-PCR

Total RNA was extracted from the LV using TRI reagent (Invitrogen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) as previously described [4, 5]. The mRNA expression levels of ANP, Collagen IV, TNF, IL-1β, IL-6, the p50 subunit of NF-κB, gp91*phox* and AT1-R were determined using previously published specific custom made primers[3-7]. GAPDH was used as the housekeeping gene. Real-time RT-PCR (qRT-PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iTaq SYBR™ Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). A dissociation step (15 s at 95°C, 15 s, at 60°C and 15 s at 95°C) was added to check the melting temperature of the specific PCR product.

HDAC activity analysis by colorimetric assay

Nuclear extracts of LV tissue were obtained with a Nuclear Extraction Kit (BioVision). Nuclear extract was then analyzed for HDAC activity with a Colorimetric HDAC Activity Assay Kit

(BioVision), both according to manufacturer's instructions. The plate was read by a Multiskan Spectrum system and values were determined as O.D./μg protein in samples.

AT1-R protein analysis by Western blot

The protein expression of AT1-R in the heart was analyzed by Western blot as previously described[4] with the use of anti-AT1-R antibody (Santa Cruz). Bands were normalized to GAPDH.

Localization of TNF and IL-1β by Immunohistochemistry

Heart tissues were prepared as previously described[5]. The sections were treated with respective primary antibodies TNF (1:100 dilution, anti-goat) and IL-1β (1:50 dilution, anti-rabbit) (Santa Cruz). Negative control sections were incubated with secondary antibody alone.

Localization of gp91*phox* by Immunofluorescence

For detection of gp91*phox* in left ventricular heart tissue, slides were incubated overnight at 4ºC with a 1:100 dilution of goat polyclonal anti-gp91*phox* (Santa Cruz) as previously described [8].

Electrophoretic Mobility Shift Assay (EMSA) for assessment of NF-kB activity

EMSA was used to assess the activity of NF-kB in the left ventricle as previously described[6].

Statistical analysis of data

All results are expressed as mean ± SEM. For statistical analysis of the data, student's *t* test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni's *post hoc* test was performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA, to determine differences among groups. Cardiac fibrosis measurements were compared by using nonparametric Kruskal-Wallis ANOVA followed by Mann-Whitney U *post hoc.* A value of $p< 0.05$ was considered statistically significant.

References

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4. Sriramula S, Haque M, Majid DS, Francis J. Involvement of tumor necrosis factor-alpha in angiotensin II-mediated effects on salt appetite, hypertension, and cardiac hypertrophy. *Hypertension.* 2008;51:1345-1351.
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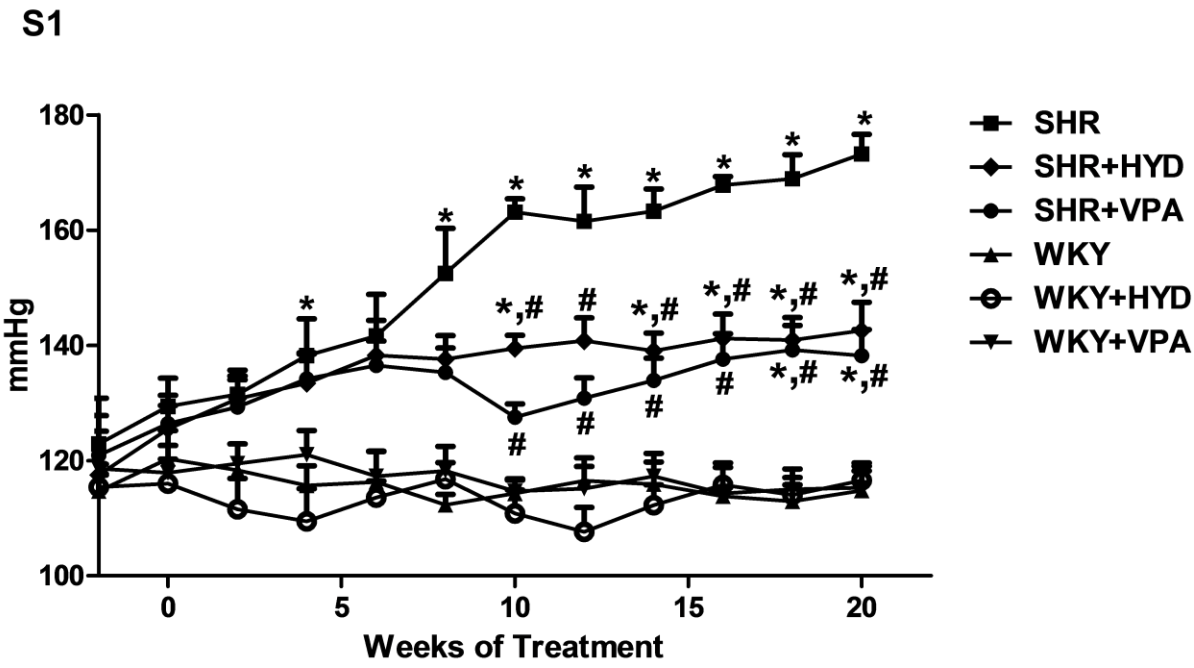
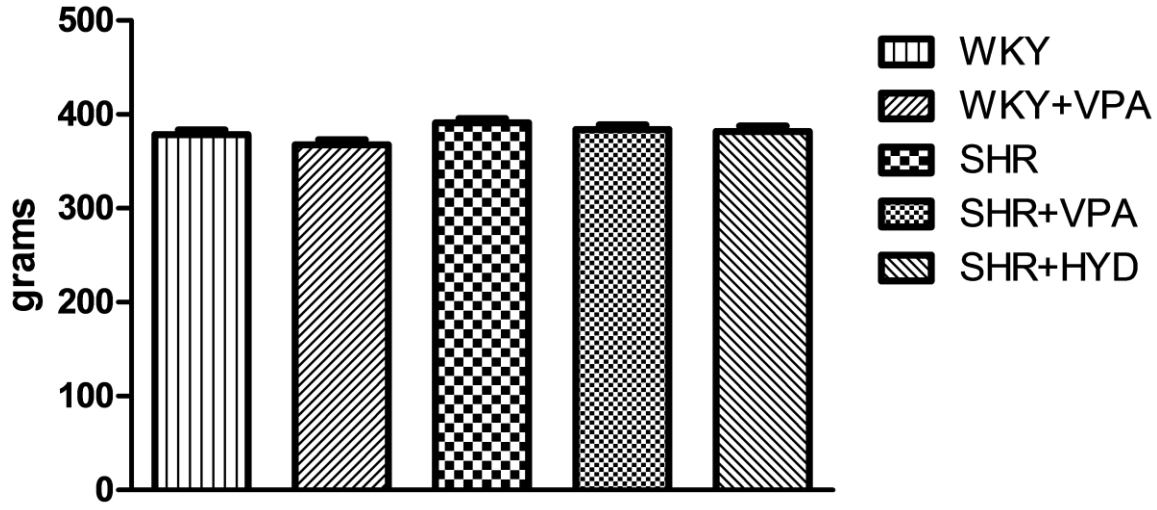
Supplemental Figures and Legends



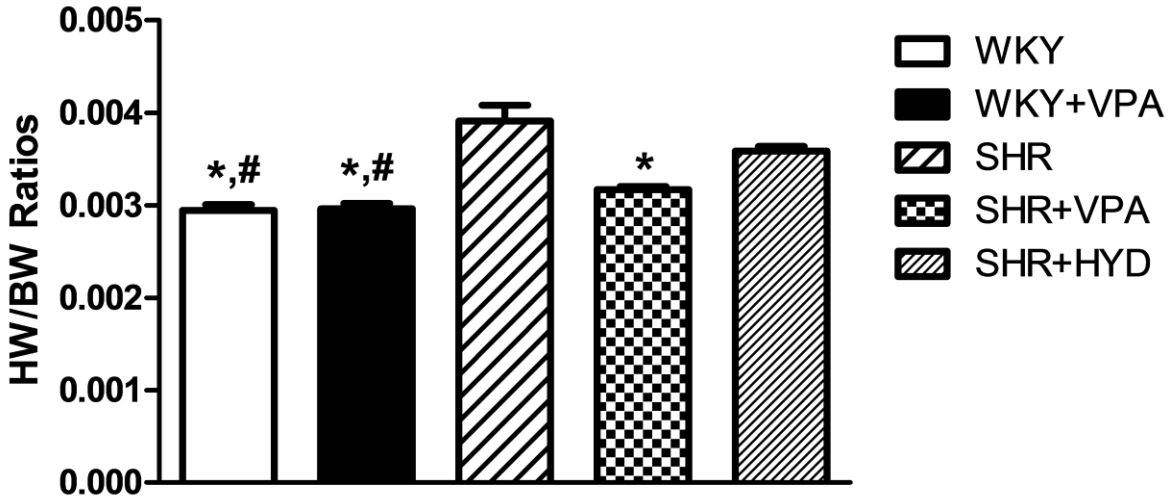
S1. Effects of VPA and HYD treatment on MAP. VPA and HYD attenuated the increase in MAP seen in untreated SHR rats. VPA: valproic acid, HYD: hydralazine, MAP: mean arterial pressure. n=7-8 in each group, *P<0.05 vs WKY, #P<0.05 vs SHR.

S2



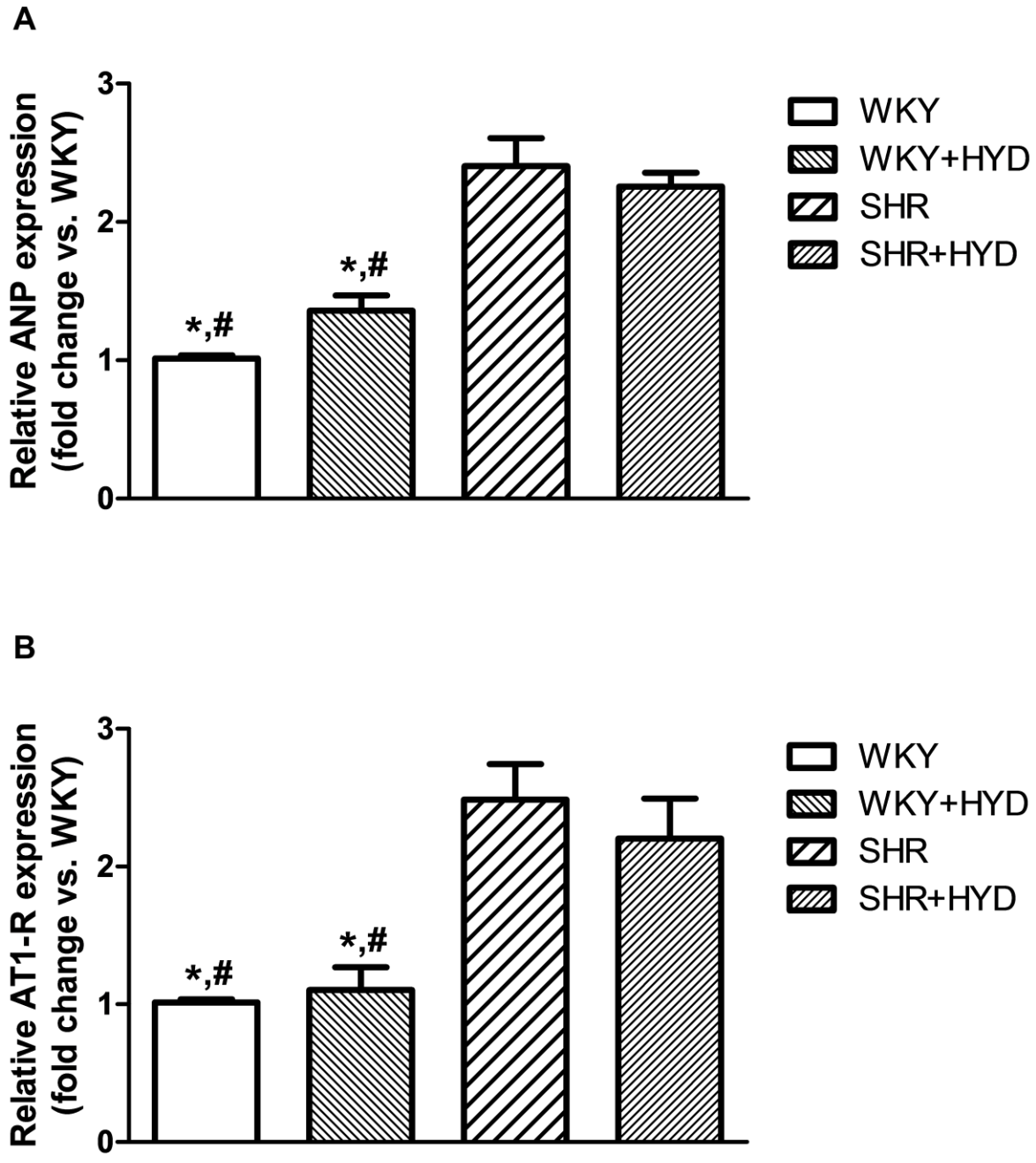
S2. Effects on VPA and HYD treatment on BW. There were no BW differences across SHR and WKY rats or between treatment groups. VPA: valproic acid, HYD: hydralazine, BW: body weight. n=7-8 in each group.

S3



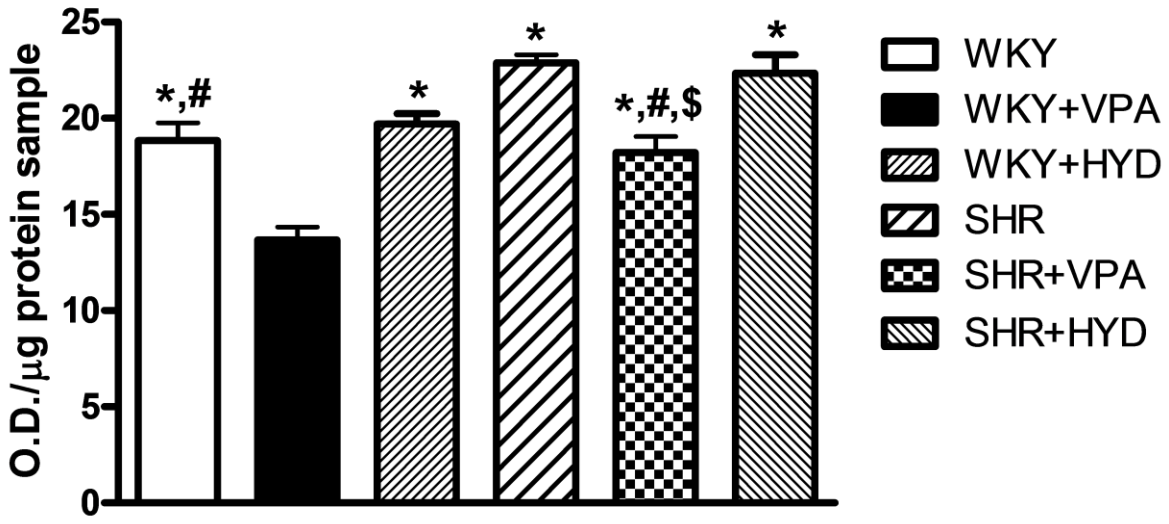
S3. Effects of VPA and HYD treatment on cardiac hypertrophy. VPA attenuated the HW/BW (indicating reduction in cardiac hypertrophy) ratio, whereas HYD had minimal effect in reducing cardiac hypertrophy in SHR rats. VPA: valproic acid, HYD: hydralazine, HW: heart weight, BW: body weight. n=7-8 in each group, *P<0.05 vs SHR, #P<0.05 vs SHR+HYD.

S4



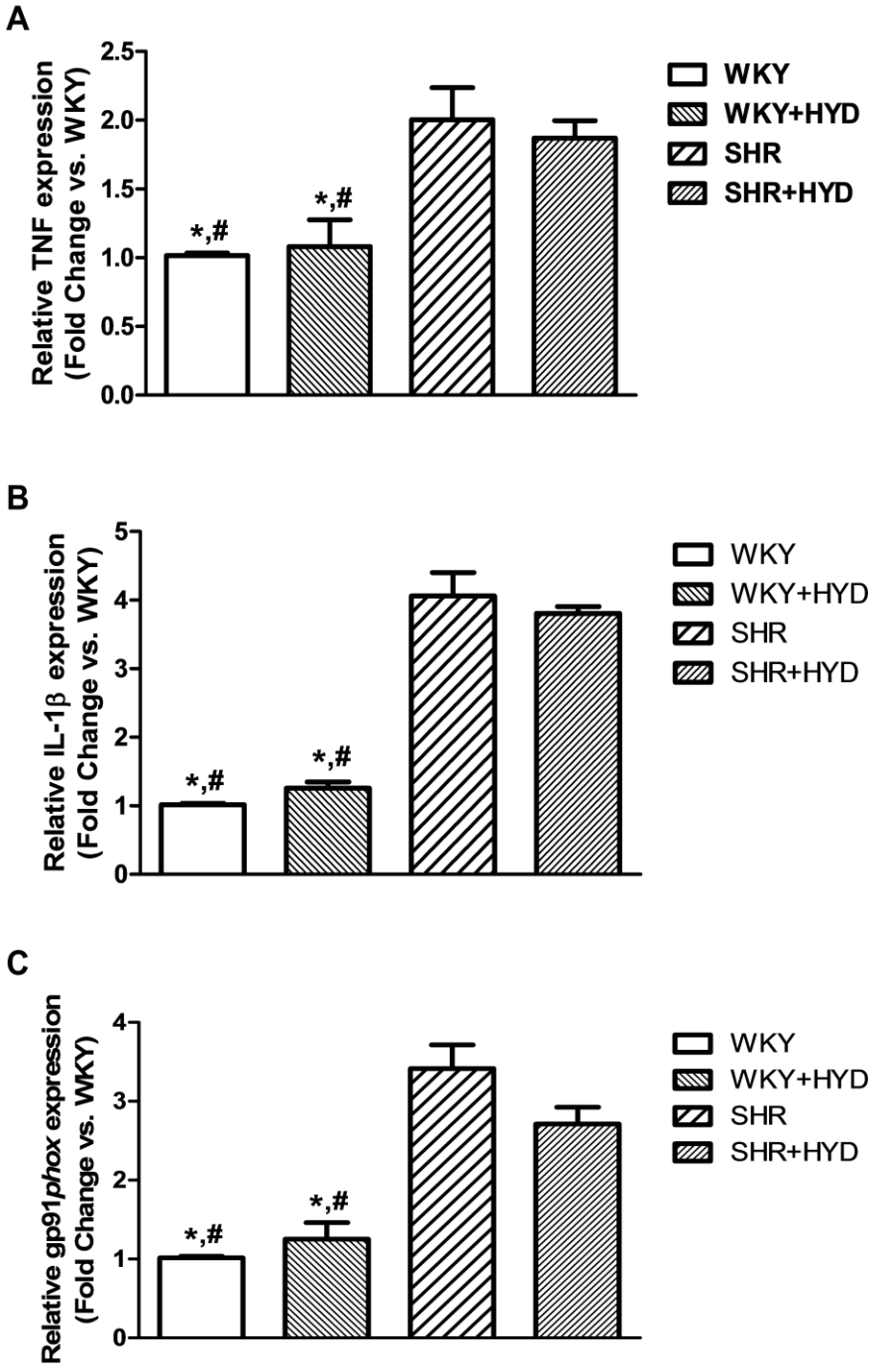
S4. Effects of HYD treatment on hypertrophic markers. SHR rats had increased levels of ANP (S4A) and AT1-R (S4B) mRNA expression compared to WKY controls. These levels remained unchanged following HYD treatment. HYD: hydralazine, ANP: atrial natriuretic peptide, AT1-R: angiotensin type 1 receptor. n=7-8 in each group, *P<0.05 vs SHR, #P<0.05 vs SHR+HYD.

S5



S5. Effects of VPA and HYD on HDAC activity as assessed through a colorimetric detection assay. HDAC activity was elevated in both untreated WKY and SHR as compared to VPA treated WKY and SHR rats. HYD had no effect on HDAC activity in LV tissue of either WKY or SHR rats. Untreated SHR HDAC activity was elevated compared to untreated WKY rats. VPA: valproic acid, HYD: hydralazine, HDAC: histone deacetylase, LV: left ventricle. n=7-8 in each group, *P<0.05 vs WKY+VPA, #P<0.05 vs SHR and $P<0.05 vs SHR+HYD.

S6



S6. Effects of HYD treatment on PIC and ROS gene expression. SHR rats had increased levels of TNF (S6A) and IL-1β (S6B) mRNA expression compared to WKY controls. These levels remained unchanged following HYD treatment. SHR rats also had elevated expression levels of gp91*phox* (S6C) when compared to WKY controls. HYD had a small but not significant effect on reducing these levels. HYD: hydralazine, TNF: tumor necrosis factor-alpha, IL-1β: Interleukin-1 beta. n=7-8 in each group, *P<0.05 vs SHR, #P<0.05 vs SHR.